



POF/GB 2004/001272



INVESTOR IN PEOPLE

The Patent Office

Concept House

Cardiff Road

Newport

South Wales

NP10 8QQ

REC'D 22 APR 2004

MFPO

POF

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

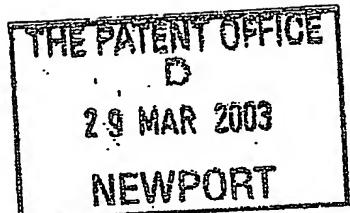
Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.



Signed

Stephen Hordley

Dated 14 April 2004



31HAR03 E796410-1 D02934
P01/7700 0.00-0307329.3

The Patent Office

Cardiff Road
Newport
South Wales
NP10 8QQ

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

1. Your reference 101009-1

2. Patent application number
(The Patent Office will fill in this part)

0307329.3

129 MAR 2003

3. Full name, address and postcode of the or of each applicant (underline all surnames)

AstraZeneca AB
SE-151 85 Sodertalje
Sweden

Patents ADP number (if you know it)

782244800

ZET

If the applicant is a corporate body, give the country/state of its incorporation

Sweden

4. Title of the invention

METHOD

5. Name of your agent (if you have one)

Neil Godfrey Alasdair PHILLIPS

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

AstraZeneca UK Limited
Global Intellectual Property
Mereside, Alderley Park
Macclesfield,
Cheshire SK10 4TG

Patents ADP number (if you know it)

8468977001

ZET

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

Country

Priority application number
(if you know it)Date of filing
(day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing
(day / month / year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:
 a) any applicant named in part 3 is not an inventor, or
 b) there is an inventor who is not named as an applicant, or
 c) any named applicant is a corporate body.
 See note (d))

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form.
Do not count copies of the same document

Continuation sheets of this form

Description 14

Claim(s) 2

Abstract 1

Drawing(s) 7 *8*

8

2

10. If you are also filing any of the following, state how many against each item.

Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (Patents Form 7/77)

Request for preliminary examination and search (Patents Form 9/77)

Request for substantive examination (Patents Form 10/77)

Any other documents
(please specify)

11.

I/We request the grant of a patent on the basis of this application.

Signature

Neil G. Phillips Date 28.03.03

Neil Godfrey Alasdair PHILLIPS, Authorised Signatory

12. Name and daytime telephone number of person to contact in the United Kingdom

Shirley Douglas - 01625 510057

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

- a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 08459 500505.
- b) Write your answers in capital letters using black ink or you may type them.
- c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.
- d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.
- e) Once you have filled in the form you must remember to sign and date it.
- f) For details of the fee and ways to pay please contact the Patent Office.

METHOD

The present invention relates to novel methods for targeting intracellular pathogens. In particular it relates to methods of inhibiting metabolic pathways essential for 5 the viability of a microorganism.

In the presence of glucose during aerobic respiration all organisms generate ATP with a concomitant release of two CO_2 molecules through the TCA/Krebs cycle. Microorganisms in addition have the unique ability to generate ATP through a shunt of the 10 TCA cycle called the glyoxylate pathway. This pathway is essential for the organism when grown in C2 carbon source like acetate. In absence of the glyoxylate bypass both the carbons of acetate would be converted to CO_2 and there would not remain any residual carbon to be utilized for the synthesis of cellular constituents. The glyoxylate bypass consists of enzymes isocitrate lyase (*icl*) and malate synthase(*ms*). In *E.coli* the bypass enzymes are induced by acetate

15 The branch point between the glyoxylate bypass and the TCA/Krebs cycle in *E.coli* is a well-studied area documenting the utility of compensatory phosphorylation.. In *E.coli* the genes *icl* and *ms* are on an operon along with the kinase *aceK*. The major control of flux at this branch point is through a reversible phosphorylation of the branch point enzyme isocitrate dehydrogenase (*idh*). The two branch point enzymes isocitrate lyase (*icl*) and 20 isocitrate dehydrogenase(*idh*) compete for the common substrate isocitrate. In the presence of glucose the large differential of K_m (*icl/idh*~80) and the relatively low concentration of isocitrate(30 μM) compared to the high K_m of *icl* (604 μM) makes all the isocitrate flow through *idh*. However in presence of two carbon substrates like acetate not only does the isocitrate concentration increase to 111 μM , more importantly a specific kinase; isocitrate 25 dehydrogenase kinase (*aceK*) is induced which phosphorylates *idh* thereby inactivating the enzyme. Under this condition a significant amount of isocitrate can flow through the glyoxylate bypass thus preventing the loss of the carbon atoms as CO_2 . The details of this control at the branch point of a metabolic pathway where the flow of substrate is ultra sensitive to factors such as the substrate concentration, the differential K_m and the reversible 30 inactivation of the branch point enzyme through phosphorylation by a specific kinase has been thoroughly worked out by Koshland and co-workers. (LaPorte, Walsh & Koshland J Biol Chem. 1984 vol259,14068-14075; Walsh & Koshland J Biol Chem. 1985,vol 260, 8430-8437))

Recently the role of the glyoxylate shunt has become the subject of intensive investigation because of the landmark work where it was shown by McKinney and co-workers (McKinney *et al.*, Nature, 2000 vol.406 735-738) that tuberculosis strains in which the *icl* gene have been inactivated were eliminated from activated macrophages at a 5 significantly higher rate than the parent strain with the wild type *icl* gene. Similarly the *icl* mutant had reduced virulence in immunocompetent animals. In contrast the *icl* mutant grew in *in-vitro* conditions as well as the wild type strain. These findings established the fact that the *icl* gene product isocitrate lysase and thus the glyoxylate pathway was essential for intracellular survival. In *M.tuberculosis* there are two *icl* genes for encoding isocitrate lyase 10 the significance of which is not understood. Sacchettini *et al.*, 2002 WO 02/33118 A2) describe and claim isocitrate lyase as the target for anti-tubercular drug development.

The genes encoding the glyoxylate shunt enzymes have also been shown to regulate pathogenicity and persistence in other intracellular pathogens. *icl* mutants of *C.albicans* (Lorenz *et al.*, Nature, 2001 vol 412, 83-86,) are unable to multiply within 15 macrophages and malate synthase mutants of the plant pathogen *Rhodococcus fascians* fail to persist in the leafy gall disease caused by the wild type (Vereecke *et al* Trends in Microbiology, 2002, vol10, 485-488) . Taken together the data support the involvement of the "glyoxylate shunt" in the intracellular survival of bacterial and plant pathogens. This may be a reflection of the type of substrates available within the environmental niche.

20 The present invention is based on the discovery that it is possible to inhibit a metabolic pathway essential for the viability of a microorganism by diverting the substrate of the pathway into a different metabolic pathway. A particular advantage is that it is not necessary to inhibit one or more enzymes in the essential pathway.

25 We call this unique mode of inactivating an enzyme/pathway "substrate diversion mechanism". It represents a novel mechanism of action in drug discovery.

Therefore in a first aspect of the invention we provide a method for attenuating a 30 microorganism, which comprises inhibiting, in the microorganism a metabolic pathway essential for viability by promoting use of the substrate of the pathway in a different metabolic pathway which is non-essential to the microorganism whereby the substrate is unavailable to the essential pathway and the microorganism is attenuated

By "metabolic pathway essential for viability" we mean a metabolic pathway that must operate for the microorganism to exist in a state other than cell death or leading to cell death. This includes cell growth, resting and latent states.

By "substrate of the pathway" we mean the only or main substrate of the pathway 5 such that the viability of the organism is dependent on the availability of this.

By "attenuating a microorganism" we mean killing it or at least decreasing its growth rate to an extent where the immune system of a human or animal host is able to contain or eliminate the microorganism. or a state, for example in a plant host, in which the 10 microbe is rendered incapable of causing disease. This may mean that metabolism via the essential metabolic pathway is either fully or partially inhibited. It may not be necessary to fully inhibit the pathway to achieve useful pharmacological effects.

In a further aspect of the invention the essentiality of the metabolic pathways is determined by the presence or absence of particular nutrients. By this we mean that the 15 microorganism may be adapted to remain viable in the absence of a particular nutrient such as glucose, glycerol or amino acids or nucleotides (in case of specific auxotrophs) by means of compensatory or shunt pathways which become essential in the absence of such nutrient and the normal pathway for metabolising such nutrient thus becomes the non-essential pathway.

The method of the invention may be used with any convenient microorganism, these include pathogens which have a restricted environment as far as nutrient options are 20 concerned like *Candida albicans* (fungal infection), *Leptosphaeria maculans* (fungal plant pathogen, Idnurm *et al*, Eukaryotic Cell 2002, vol 1. 719-724) & *Rhodococcus fascians* (plant pathogen) This is strikingly exemplified in many pathogens whose pathogenicity is dependent on intracellular survival and multiplication, a stage closely associated with the persistence or latency stage of *Mycobacterium tuberculosis*. The list of organisms which encompass such 25 stage is given in Table 1 hereinafter.

The person of ordinary skill will be able to select convenient essential and non-essential metabolic pathways and substrates for use in the method of the invention. By way of non-limiting example it is convenient to target metabolic branch points. At such points enzymes from the two metabolic pathways compete for the common substrate. Control at 30 branch points may depend on a number of factors including the growth medium, substrate concentration, the differential Km and the reversible inactivation of enzymes beyond the branch point.

It is known that a branch point in a metabolic pathway provides a unique mode of control of substrates through a pathway. One of the first studies on branch point effect and disease was done on gout and regulation of purine biosynthesis(Hershfield & Seegmiller, Horiz Biochem Biophys. 1976;vol 2:134-62.

5 Arachidonic acid is the branch point substrate between two families of potent physiological effectors, one including cyclooxygenase which leads to the synthesis of prostaglandins and thromboxanes, the other involving lipoxygenase which leads to the synthesis of leukotrienes and lipoxins. While aspirin and other NSAID are available as potent inhibitors of cyclooxygenase no effective inhibitor of lipoxygenase are available (Prigge *et al*
10 Biochimie 1997, vol 79, 629-636)

The target for anticancer therapy IMP dehydrogenase(IMPDH) is at the metabolic branch point of purine nucleotide synthesis pathway. Blockage of the conversion of IMP to XMP by IMPDH inhibitors lead to a depletion of the guanylate (GMP,GDP,GTP and dGTP pools) leading to the death of the rapidly dividing cancer cells (Franchetti & Grifantini, Curr.
15 Med.Chem. 1999 , vol6, 599-614). Computer simulation of simple biochemical pathways containing divergent branch has been used to study the interaction of two inhibitors that straddle the branch point (Jackson, Cancer Res.1993 vol. 53, 3998-4003). However to the best of our knowledge the concept of substrate diversion for inhibition of a branched pathway has not been anticipated.

20 Promoting use of the substrate in the non-essential metabolic pathway may be achieved by restoring function to one or more enzymes in the pathway. In a typical branch point where one branch leads to an essential pathway while the other branches to a non-essential one, the essential pathway may be activated by diverting the substrate through the non essential path using the control parameters mentioned earlier.. This strategy may also be
25 used for pathways having more than two branches. Some of the metabolic pathways which have branch points are the Glyoxylate pathway, Glycolysis, Pentose Phosphate pathway, Purine Salvage pathway and Phenylalanine and Tyrosine metabolism.

A particular essential metabolic pathway is the glyoxylate pathway in the TCA/Krebs cycle. As previously mentioned microorganisms have the unique ability to
30 generate ATP through the glyoxylate pathway.

The flow of the substrate isocitrate is generally through the TCA cycle when the organism grows in glucose. However as mentioned in the prior art there is a partial diversion through the glyoxylate bypass when the carbon source is acetate (C2 carbon source). This is

outlined in Figure 1. This regulation which depends on the Km isocitrate for *icl* and *idh*, its velocity and the phosphorylation of *idh* by *aceK* has been elaborately worked out by Koshland and co-workers (LaPorte, Walsh & Koshland J Biol Chem. 1984 vol259, 14068-14075; Walsh & Koshland J Biol Chem. 1985, vol 260, 8430-8437). It has also been shown that *aceK* mutant strain of *E.coli* grows well like the wild type strain when the carbon source is C6 like glucose. In contrast it is starved to death under C2 carbon source (LaPorte, Thorsness & Koshland J.Biol. Chem1985, vol26, 10563-10568). This indicates the essentiality of substrate diversion.

By way of non-limiting example the glyoxylate pathway has been found in the following organisms:

E.coli, *S.typhi*, *S.typhimurium*, *Y pestis*, *S.flexneri*, *X.fastidiosa*, *X.campestris*, *X.axonopodis*, *V.cholerae*, *V.vulnifucus*, *P.aeruginosa*, *P.putida*, *S.onedensis*, *R.solanacearum*, *M.lotii*, *S.meliloti*, *A.tumefaciens*, *B.meliloti*, *B.suis*, *B.japonicum*, *C.crescentus*, *O.iheyensis*, *M.tuberculosis*, *M.leprae*, *C.glutanicum*, *C.efficiens*, *S.coelicolor*, *D.Radiodurans*, *S.solfataricus*, *C.elegans*, *A.thaliana*, *S.cervisiae*

For the glyoxylate shunt to operate analogous to that observed in *E.coli*, the trio of genes isocitrate dehydrogenase (*icd*), isocitrate lyase(*icl*) and isocitrate dehydrogenase kinase(*icdk*) should be present. Though *icd* and *icl* have been annotated in a large number of organisms *icdk* has only been identified in a few. The list given below in Table 1 names the organisms where the trio of the genes have been identified. These genes (with its id) were identified from the KEGG database.(www.genome.ad.jp/kegg)

Table 1

Organism	<i>icd</i>	<i>icl</i>	<i>Icdk</i>
<i>E.coli</i>	b1136	b4015	b4016
<i>E.coliJ</i>	JW1122	JW3975	JW3976
<i>E.coli0157</i>	Z1865	Z5601	Z5602
<i>E.coli0157J</i>	Ecs1608	Ecs4933	ECs4934
<i>E.coliCFT073</i>	c1517	c4972	c4974
<i>S.typhi</i>	STY1278	STY4402	STY4403
<i>S.typhimurium</i>	STM1238	STM4184	STM4185
<i>Y pestis</i>	YPO1641	YPO3725	YPO3724

<i>Y. pestis</i> KIM	y1802	y0016	y0018
<i>S. flexneri</i>	SF1155	SF4081	SF4082
<i>X. campestris</i>	XCC0967	XCC0238	XCC3780
<i>X. axonopodis</i>	XAC1046/3835	XAC0257	XAC3832
<i>P. aeruginosa</i>	PA2623	PA2634	PA1376
<i>P. putida</i>	PP4012	PP4116	PP4011/4565
<i>R. solanacearum</i>	RSO1106	RSO4640	RSO3245

In a particular aspect of the invention the microorganism is *Mycobacterium tuberculosis*. As mentioned earlier the glyoxylate bypass of this bacteria has been proposed to be essential for its intracellular survival within the macrophage. In order to 5 target the glyoxylate bypass of *M. tuberculosis* by the mechanism of substrate diversion as mentioned earlier it is essential to identify the target enzyme responsible for the diversion, the *M. tuberculosis* equivalent of idh-kinase. This enzyme is a unique target for inhibiting through the method of the invention as cells inhibited of this enzyme would not be able to survive within the macrophage. As this kinase has not previously been identified in *M. tuberculosis*, 10 we describe below in the Examples the approach we have employed to identify and characterise this enzyme as *pknG*.

In a further aspect of the invention we disclose a method for identifying compounds that attenuate *Mycobacterium tuberculosis* which method comprises testing compounds in a test system for their ability to bind to *pknG* and prevent autophosphorylation 15 of *pknG*.

In a further aspect of the invention we disclose a method for identifying compounds that attenuate *Mycobacterium tuberculosis* which method comprises testing compounds in a test system for their ability to bind to *pknG* and prevent phosphorylation of *icd1*.

20 In a further aspect of the invention we disclose a method for identifying compounds that attenuate *Mycobacterium tuberculosis* which method comprises testing compounds in a test system for their ability to bind to *icd1* and prevent phosphorylation of *icd1* by *pknG*.

25 In a further aspect of the invention we disclose a method for identifying compounds that attenuate *Mycobacterium tuberculosis* which method comprises testing

compounds in a test system for their ability to prevent the phosphorylation and or inactivation of *icd1*.

Convenient test systems for use in the above methods will be apparent to the scientist of ordinary skill. By way of non-limiting example such test systems will 5 conveniently comprise as appropriate either the *pknG* enzyme or the *icd1* enzyme or both enzymes, the enzyme may be coupled to a reporter system capable of indicating as appropriate the presence or absence of autophosphorylation of *pknG* or phosphorylation of *icd1*. Since the phosphorylation of *icd1* will lead to its concomitant inactivation, the degree of phosphorylation can be indirectly measured by monitoring its activity in 340nm. which 10 measures the formation of NADPH (Figure 7). Using radiolabelled (32 γ) ATP we can monitor the phosphorylation of *PknG* and/or *icd1* (Figure 6). Also the degree of phosphorylation of either *PknG* and *icd1* can be directly measured in a standard ELISA format by using either generic phosphoserine antibodies or antibodies specific to either of the enzymes.

In a further aspect of the invention we provide an antimicrobial compound 15 identified by one of the above method aspects of the invention.

The invention will now be illustrated but not limited by reference to the following non-limiting Examples and Figures wherein:

Figure 1 illustrates the common isocitrate substrate for the *idh* enzyme in the TCA cycle and 20 the *icl* enzyme in the glyoxylate bypass. *Idh-kinase* inactivates *idh* and thus turns on the glyoxylate bypass.

Figure 2 shows a neighbour joining tree for the 11 *M. tuberculosis* Ser-Thr protein kinases.

25 Figure 3 shows an average tree for the 11 *M. tuberculosis* Ser-Thr protein kinases.

Figure 4 illustrates the purification of *pknG*. Lane 1 and 2 indicate the purified *pknG* (indicated by the arrow). Lane M indicates the molecular weight markers.

30 Figure 5 illustrates the purification of isocitrate dehydrogenase. The purified protein was monitored by 10% SDS-PAGE and is shown as a 45.4 Kda band.

Figure 6 shows the results of experiments to show that pknG specifically phosphorylates icd1. The results show that pknG specifically phosphorylates icd1 (lane 1) whereas when E.coli icd is added as a substrate, only autophosphorylation of pknG occurs (lane 2).

5 Figure 7 shows a graph of the typical reaction of icd1 in the presence and absence of pknG. The y-axis shows absorbance as measured at 340nm and the x-axis shows time in seconds.

Example 1Protein kinases in *M. tuberculosis*; identification of *pknG* as the idh-kinase in *M. tuberculosis*

The protein kinase 'signature', including all 11 domains that are conserved 5 according to Hanks (Hanks, S. K., A. M. Quinn, and T. Hunter. *Science*, 1988 vol. 241: 42-51) is present in all the eleven *M. tuberculosis* Ser-Thr protein kinases (STPK) that have been annotated. An additional gene, *pknM*, which was previously annotated as a putative STPK-encoding gene, does not possess any recognizable kinase signatures. The homology between *pknM* and the carboxy-terminal region of *pknH* probably explains the original annotation. 10 Alignment of the STPK family members revealed that 15 residues are absolutely conserved across the group. All kinases other than *pknI* possess a lysine in the active site [Hanks domain VIb (DXKPXN, where X is any amino acid)], which is characteristic of STPKs. *pknI* has an asparagine at this position, which is unusual for an STPK; however, the other conserved residues in the domain indicate that *pknI* is a Ser/Thr kinase.

15 In order to identify the putative idh-kinase in *M. tuberculosis* a search strategy based on the following assumption was adopted.

1. The sequence alignment of all the eleven STPK of *M. tuberculosis* was compared with the idh-kinase of *E. coli* (*aceK*).
2. Since the idh-kinase activity occurs in conjunction with all the TCA cycle enzymes, it should occur in the cytosol, the enzyme should lack transmembrane domains. 20
3. The specific idh-kinase of *M. tuberculosis* should have least homology with the other STPKs.
4. It is presumed that a homolog of the putative idh-kinase in *M. tuberculosis* is also present in *M. leprae*, as it is also a intracellular pathogen.

25 Homology with *aceK*

A bioinformatics approach was taken to identify the putative homologous kinase among the annotated *M. tb* STPKs with *aceK* of *E. coli*. The effort was not restricted within the sequence analogy or motif searching but rather took advantage of the known relationship between the STPK's of *M. tb* and the structural homology displayed between *aceK* and eukaryotic STPKs. The main features identified were the followings; 30

The strongly and weakly homologous residues differ among the 12 sequences. Based on the strongly homologous residues K and L in the second conserved motif and the weakly homologous residue A in the third conserved motif, it is seen that *pknG*, *pknL*, *pknA* and *pknB* are most similar to *E. coli aceK*.

5

Transmembrane Domains(TM) in *M. tuberculosis* STPKs.

Gene	Rv No:	TM domain
PknA	0015c	+
PknB	0014c	+
PknD	0931c	+
PknE	1743c	+
PknF	1746c	+
PknG	0410c	
PknH	1266c	+
PknI	2914c	+
PknJ	2088c	+
PknK	3080c	
PknL	2176c	+

Table 2.

10 It is seen from Table 2 that the TM domain is absent in the genes *pknG* and *pknK* indicating that probably these two STPKs act in the cytosolic milieu.

Homology and clustering of the STPKs

Table 3

From the above homology matrix (Table 3) it is seen that the genes *pknG* and *pknK* are least homologous with other STPKs. However the general homology among the STPKs being low it was decided to look at how the genes cluster among each other when the clustering is done by near neighbour tree and average tree algorithm.

From the homology and clustering of the *M.tuberculosis* STPKs (Figure 2 and Figure 3) it seems that the genes *pknG* and *pknK* are outliers when compared to other STPKs.

STPKs in *M.leprae*

Compared to *M.tuberculosis* nearly half of the genes in *M.leprae* are either absent or pseudogenes. It is postulated that only the bare essential genes are present in *M.leprae* (Brosch R, Gordon S V, Eiglmeier K, Garnier T, Cole S T. Comparative genomics of the leprosy and tubercle bacilli. Res. Microbiol 2000; 151:135–142). Of the 11 STPKs only four, *pknA*, *pknB*, *pknG* and *pknL* are present in *M.leprae*.

The above analysis strongly indicates that when all the factors, like homology to *acek*, absence of transmembrane domain, outlier in STPKs, and presence in *M.leprae* are taken into account only *pknG* satisfies all the four characteristics. Thus we are satisfied that *pknG* is the icd-kinase in *M.tuberculosis*.

Example 2

Phosphorylation of icd by *pknG* in *M.tuberculosis*

In order to demonstrate that *pknG* is the specific icd-kinase in *M.tuberculosis* we decided to show that it specifically phosphorylates the isocitrate dehydrogenase of *Mycobacterium tuberculosis*. Additionally this phosphorylation also inactivates the enzymic activity of *icd*. To this end we have cloned, expressed and purified *pknG* and *icd*.

Cloning and Purification of *pknG*:

The gene encoding *pknG* (Rv 0410c) was PCR amplified and cloned into the expression vector pET 21D. The recombinant plasmid was then transformed into the host BL21(DE3). Following induction by 1mM IPTG for 2hrs the cells were collected by centrifugation and lysed by sonication in Tris buffer (pH 7.5). Following sonication the cell lysate was centrifuged at 30000Xg. It was seen that the protein was expressed in the soluble

supernatant. Following this the protein was precipitated in 35% ammonium sulphate. The protein pellet was then dissolved in Tris buffer and purified through anion exchange (Q-Sepharose) chromatography. It was seen (Figure 4) that *pknG* eluted around 300nM NaCl. The purity of the protein was checked in 10% SDS-PAGE.

5 Cloning and Purification of isocitrate dehydrogenase:

There are two gene sequences (Rv 0066c and Rv3339c) annotated as isocitrate dehydrogenase in *M.tuberculosis*. However none of them have been proven experimentally to be the said enzyme. The smaller of the two genes *icd1* (Rv 3339c) whose size is comparable to the *E.coli* gene was PCR amplified and cloned into the expression vector pET8c. The recombinant plasmid was then transformed into the host BL21(DE3). Following induction by 1mM IPTG for 2hrs the cells were collected by centrifugation and lysed by sonication in Tris buffer(pH 7.5). Following sonication the cell lysate was centrifuged at 30000Xg. It was seen that the protein was expressed as inclusion bodies. The inclusion bodies were then purified in step (15 & 60%) sucrose gradient. The purified inclusion bodies were then solubilized in 6M GnHCl and refolded by rapid dilution in PBS in presence of 1M NDSB. The purified protein was then monitored by 10% SDS-PAGE shown below

Specific phosphorylation of *icd1* by *pknG*

To prove that *pknG* specifically phosphorylates *icd1* we set up a kinase reaction in a buffer (Tris~pH 7.4 containing 5mM MgCl₂, 2mM MnCl₂, 0.1mM DTT and 0.1mM Na Orthovanadate) the transfer molecule ATP was 0.1mM in presence of $\gamma^{32}P$ ATP. The amount of proteins, *pknG* and *icd1*, in the reaction was 1 μ g and 2.5 μ g respectively. As a negative control *E.coli* *icd* protein was also used in a separate reaction. Following the kinase reaction the products were run on a SDS-PAGE and autoradiographed. The results are shown in Figure 6.

25 The results clearly indicate that *pknG* specifically phosphorylates *icd1* (lane1) whereas when *E.coli* *icd* is added as a substrate, only autophosphorylation of *pknG* occurs (lane2).

Inactivation of *icd1* due to phosphorylation by *pknG*

So far we have shown that *pknG* specifically phosphorylated *icd1* of *M.tuberculosis*. However in order for the branch point substrate diversion to occur it has to be shown that phosphorylation of *icd1* causes its inactivation. In an earlier experiment it was shown that in *pknG* it is the serine residue that is phosphorylated (Koul *et.al* Microbiology

2001, vol147,2307-2314) hence it would be logical to assume that phosphorylation of *icd1* by *pknG* occurs at the serine residue. The reaction catalysed by *icd1* in the absence is be given by:



5 By measuring the formation of NADPH at 340nm we can monitor this reaction. However if the phosphorylation of *icd1* by *pknG* causes the inactivation of the former then the formation of NADPH is reduced thereby reducing the absorbance at 340nm. Shown below in Figure 7 is the typical reaction of *icd1* in presence and absence of *pknG*. It is obvious from the above result that phosphorylation of *icd1* by *pknG* results in a 10 concomitant inactivation of the former.

A Km DRIVEN SHUNT IN *MYCOBACTERIUM TUBERCULOSIS*

There are two important features of the glyoxylate bypass enzymes which enables it to be operative in a micro organism. The first is the phosphorylation dependent inactivation 15 of the *idh*. This, as we have shown above by the specific phosphorylation of *idh1* by *pknG* to be specifically operative in *M.tb*. The second is the differential Km for the substrate isocitrate between *idh* and *icl*. When the organism grows in glucose the preference of isocitrate for *idh* is governed by its differential Km. In order to verify if this is also true in *M.tb* we measured the Km of isocitrate for *idh1*. The Km as evaluated by Lineweaver-Burke 20 plot and Cornish-Bowden direct linear plot indicated a value of 12μM for isocitrate. The Km for *icls* have been previously determined by Russel and co-workers to be around 115 and 1500 μM for *icl1* and *icl2* respectively. This data clearly indicate that *idh1* has a greater affinity to isocitrate over *icls*. This data proves the presence of a differential Km and we can thus conclude the possibility of a km driven shunt to be operative in *M.tuberculosis*.

-25 In summary we disclose a novel method of pathway inactivation by substrate diversion. This concept is exemplified in *M.tuberculosis* by identification of a novel isocitrate dehydrogenase kinase (*pknG*) which specifically phosphorylates and inactivates its cognate isocitrate dehydrogenase(*icd1*). Thus inhibitors of *pknG* (compounds that bind such that the transfer of phosphate moiety to the corresponding serine residue in *icd1* is prevented), will 30 divert the substrate isocitrate away from the glyoxylate shunt. This will prevent the growth of the bacteria in C2 carbon source, which might be catastrophic when the bacteria are in the latent/persistence phase. It should be noted that *pknG* transfers the phosphoserine to *icd1* by two-step procedure, in the first step the kinase is autophosphorylated and in the second step the

phosphate is transferred. There may be two types of inhibitors, the first that prevents autophosphorylation, the second that prevents the transfer. We believe that both the type of inhibitors will prevent the phosphorylation of *icd1*.

Claims:

1. A method for attenuating a microorganism which comprises inhibiting in the microorganism a metabolic pathway essential for viability, by promoting use of the substrate of the pathway in a different metabolic pathway which is non-essential to the microorganism whereby the substrate is unavailable to the essential pathway and the micro organism is attenuated.
2. A method as claimed in claim 2 and wherein the presence or absence of a nutrient determines whether a metabolic pathway is essential or non-essential to the microorganism.
3. A method as claimed in claim 1 and wherein use of the substrate in the non-essential pathway is promoted by inhibition of an enzyme not in the essential pathway.
- 15 4. A method as claimed in claim 4 and wherein the enzyme to be inhibited is a kinase.
5. A method as claimed in claim 4 and wherein the enzyme to be inhibited is isocitrate dehydrogenase (icd1).
- 20 6. A method as claimed in claim 1 and wherein the microorganism is *Mycobacterium tuberculosis*.
7. A method as claimed in claim 6 and wherein the enzyme to be inhibited is *pknG*.
- 25 8. A method for identifying compounds that attenuate *Mycobacterium tuberculosis* which method comprises testing compounds in a test system for their ability to bind to *pknG* and prevent autophosphorylation.
9. A method for identifying compounds that attenuate *Mycobacterium tuberculosis* 30 which method comprises testing compounds in a test system for their ability to bind to *pknG* and prevent phosphorylation of *icd1*.

10. A method for identifying compounds that attenuate *Mycobacterium tuberculosis* which method comprises testing compounds in a test system for their ability to bind to *icd1* and prevent phosphorylation of *icd1* by *pknG*.

5 11. A method for identifying compounds that attenuate *Mycobacterium tuberculosis* which method comprises testing compounds in a test system for their ability to prevent the phosphorylation and or inactivation of *icd1*.

10 12. An anti-microbacterial compound identified according to the method of any one of claims 8-11.

A B S T R A C T

METHOD

5 A method for attenuating a microorganism which comprises inhibiting in the microorganism a metabolic pathway essential for viability, by promoting use of the substrate of the pathway in a different metabolic pathway which is non-essential to the microorganism whereby the substrate is unavailable to the essential pathway and the micro organism is attenuated.

10

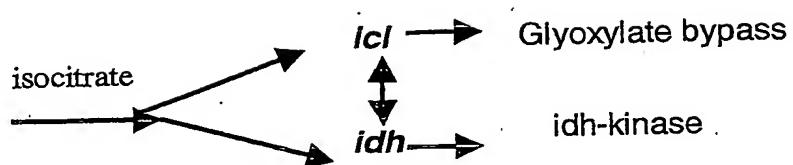
Figure 1

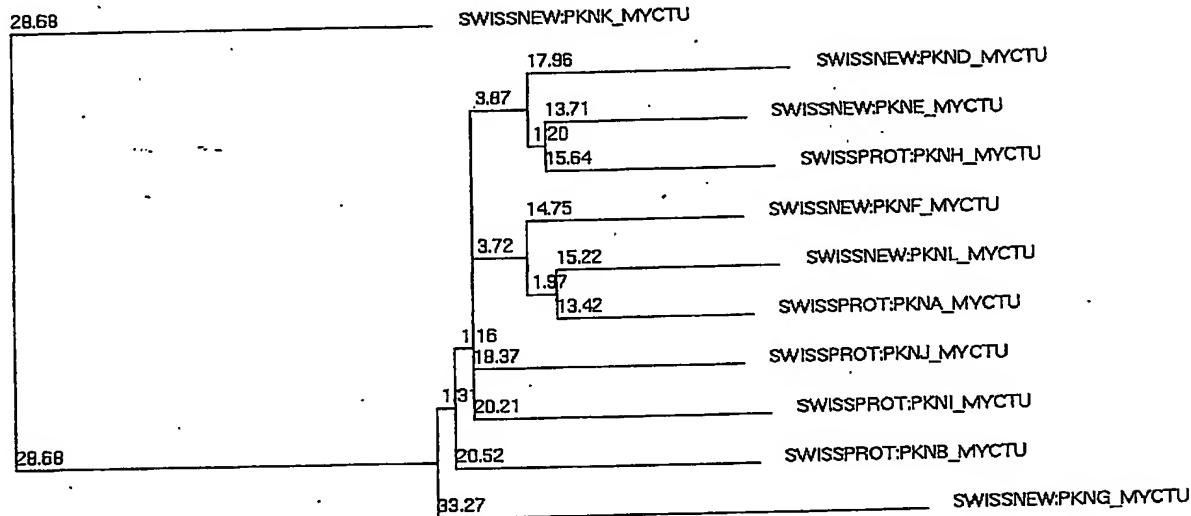
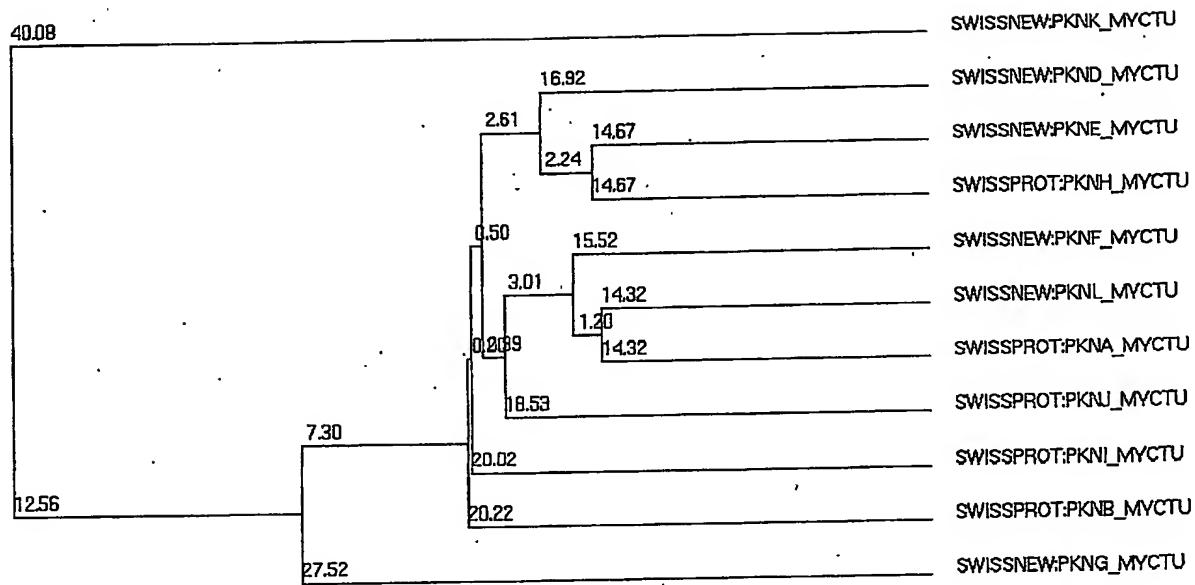
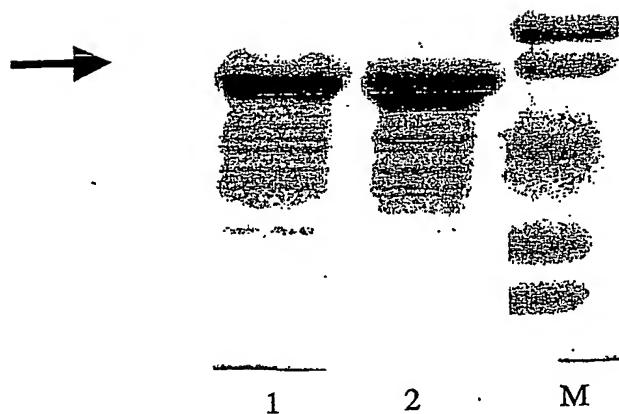
Figure 2NEIGHBOUR JOINING TREE OF *M. Tb* STPKs

Figure 3AVERAGE TREE OF *M.tb* STPKs

101009

-4/7-

Figure 4



101009

-5/7-

Figure 5

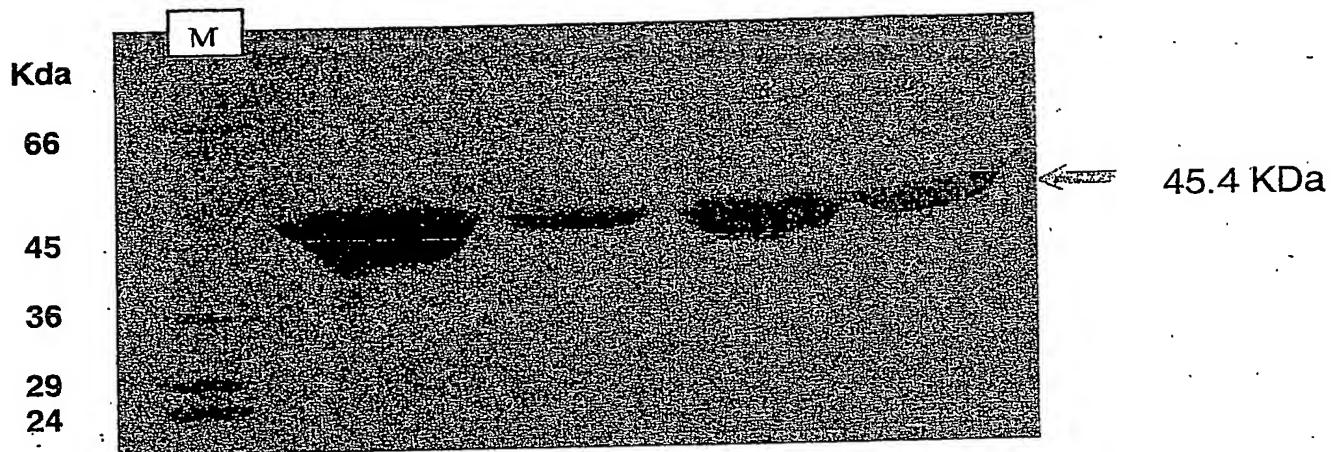


Figure 6

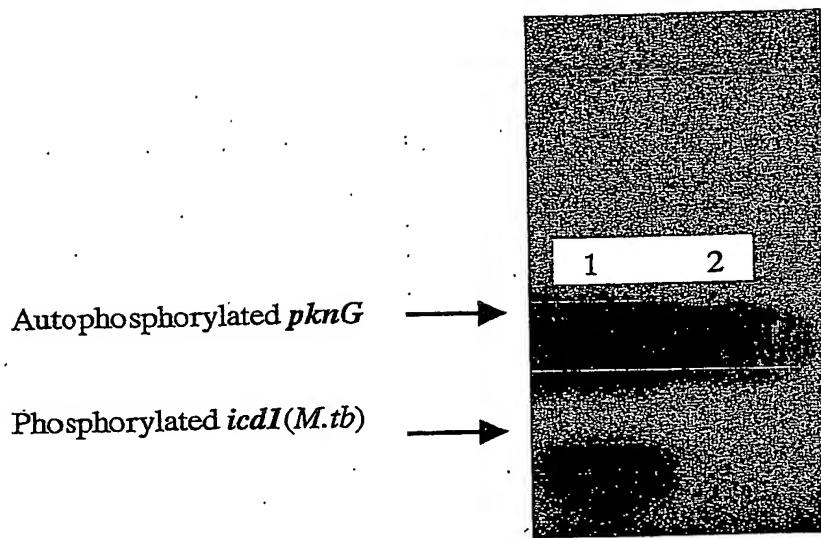
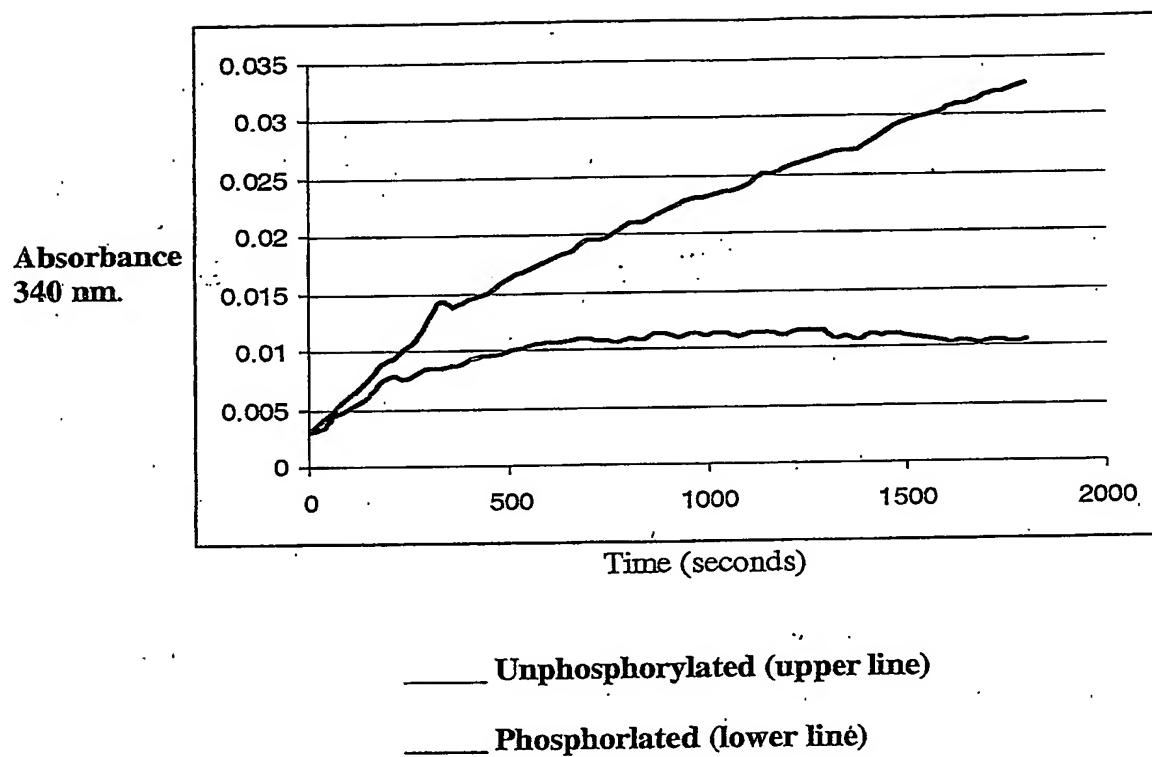


Figure 7

POST/GB2004/001272

